REVIEW

MicroRNAs as biomarkers of diabetic retinopathy and disease progression

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Abstract

Diabetes mellitus, together with its complications, has been increasing in prevalence worldwide. Its complications include cardiovascular disease (*e.g.*, myocardial infarction, stroke), neuropathy, nephropathy, and eye complications (*e.g.*, glaucoma, cataracts, retinopathy, and macular edema). In patients with either type 1 or type 2 diabetes mellitus, diabetic retinopathy is the leading cause of visual impairment or blindness. It is characterized by progressive changes in the retinal microvasculature. The progression from nonproliferative diabetic retinopathy to a more advanced stage of moderate to severe nonproliferative diabetic retinopathy and proliferative diabetic retinopathy occurs very quickly after diagnosis of mild nonproliferative diabetic retinopathy. The etiology of diabetic retinopathy is unclear, and present treatments have limited effectiveness. Currently diabetic retinopathy can only be diagnosed by a trained specialist, which reduces the population that can be examined. A screening biomarker of diabetic retinopathy with high sensitivity and specificity would aid considerably in identifying those individuals in need of clinical assessment and treatment. The majority of the studies reviewed identified specific microRNAs in blood serum/plasma able to distinguish diabetic patients with retinopathy from those without retinopathy and for the progresion of the disease from nonproliferative diabetic retinopathy to proliferative diabetic retinopathy. In addition, certain microRNAs in vitreous humor were dysregulated in proliferative diabetic retinopathy compared to controls. A very high percentage of patients with diabetic retinopathy develop Alzheimer's disease. Thus, identifying diabetic retinopathy by measurement of suitable biomarkers would also enable better screening and treatment of those individuals at risk of Alzheimer's disease.

Key Words: *diabetes; retinopathy; diagnosis; disease progression; microRNAs; biomarkers; blood serum/plasma; vitreous humor; humans*

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Introduction

Diabetes mellitus (DM) is increasing in prevalence worldwide especially in developing countries (World Health Organization, 2018). The prevalence of DM in adults aged 20–79 years was estimated to be 8.8% in 2015 and forecast to increase to 10.4% in 2040 (Ogurtsova et al., 2017). While DM can be treated, and its consequences prevented or slowed with diet, physical activity, and medication, its complications have been increasing in prevalence. These complications include cardiovascular disease (*e.g.*, myocardial infarction, stroke), neuropathy, nephropathy, and eye complications (*e.g.*, glaucoma, cataracts, retinopathy, macular edema) (American Diabetes Association; NIH National Eye Institute, 2015; Solomon et al., 2017). The primary factor in the development of diabetic complications is usually considered to be lasting exposure to hyperglycemia (Aronson and Rayfield, 2002).

In patients with either type 1 DM (T1DM) or type 2 DM (T2DM), diabetic retinopathy (DR) is the leading cause of visual impairment or blindness. It is characterized by progressive changes in the retinal microvasculature (Cheung et al., 2010; Hammes et al., 2011; Yau et al., 2012). Currently DR affects approximately 150 million people worldwide and the number is likely to double by 2025 according to the World Health Organization (King et al., 1998; Gupta et al., 2013). As the first sign of diabetic complications, DR has been used as an indicator for the diagnosis of DM complications (Genuth et al. 2003). Research evidence indicates that nearly all patients with T1DM and 60% of patients with T2DM develop some degree of retinopathy within 20 years after diagnosis (Klein et al., 1989). Similarly, it was reported that DR affects up to 80% of all patients who have had DM for ≥ 20 years (Kempen et al., 2004). In a large prospective cohort study followed for over 10 years, 45% of patients with T2DM developed any type of DR (Yun et al., 2016). At the first diagnosis of nonproliferative DR (NPDR), the mean duration of diabetes was 14.8 years, but progression to a more advanced stage of DR (moderate to severe NPDR and proliferative DR (PDR)) was very fast, occurring within about 2 to 3 years after diagnosis of mild NPDR. These data highlight the need for early detection of DR to reduce the high morbidity of this disease. While major efforts have been made to elucidate the pathomechanism of DR, the exact causes remain largely unclear, and present treatments have limited effectiveness (Stitt et al., 2016). The prevalence of DR will continue to increase due to the increased lifespan and longer duration of diabetes in DM patients.

General risk factors for the occurrence and progression of DR are longer duration of diabetes, hyperglycemia, hypertension, poor glycemic control, and dyslipidemia. While these risk factors are helpful in stratifying a patient's risk for developing retinopathy, many patients without these

traditional risk factors develop DR. Moreover, there are individuals with long diabetes duration who do not develop DR (Smio-Servat et al., 2016; Ting et al., 2016). In 2002, it was reported that 29% of diabetic patients develop DR, whereas 22% of individuals with a history of diabetes do not develop DR, regardless of glycemic exposure, indicating that genetic factors may be important in the development of DR (Cai and Boulton, 2002). Thus, identifying biomarkers to predict DR or to determine therapeutic response is important. An easily accessible, screening biomarker of DR with high sensitivity and specificity would aid considerably in detecting those individuals in need of clinical assessment and treatment (Pusparajah et al., 2016).

MicroRNAs are a major class of short (~22 nt) non-coding RNAs that function to block protein translation and/or degrade their messenger RNA targets. They bind to complementary sequences in the 3′-UTR (untranslated) region of the target messenger RNA (Krishnan and Damaraju, 2018). These small RNAs direct many important processes related to cellular growth, apoptosis, differentiation, metabolism and the immune response (Rodrigues et al., 2018). MicroRNAs (miRNAs) are involved in DR-related microvascularization, and miRNAs that exhibit increased or decreased expression during DR pathogenesis have been identified (Mastropasqua et al., 2014). Modulation of miRNA levels may be able to reverse dyslipidemia (Davalos and Fernandez-Hernando, 2013) and slow DR progression (Gong and Su, 2017), and could potentially be used in DR therapeutic strategies. However, few studies have investigated the progressive changes in miRNA levels in DR (Gong and Su, 2017). In an article by Joglekar et al. (2016) the authors wrote "*We hope that this important report of Zampetaki et al. is followed by clinical studies in which miRNAs are evaluated in larger study cohorts, across different ethnic groups, in different types of diabetes, and in a wide age range as well as different stages of DR. It would also be of interest to see small RNA-sequencing analyses from such trials and to follow up these or other identified miRNAs in longitudinal studies to determine their predictive value for DR progression and responses to treatments. As miRNAs themselves may be therapeutic targets or even therapeutic agents (as anti-miRNAs), further studies will help in identifying and assessing their therapeutic potential for the treatment of retinopathy in individuals with diabetes*". Additionally, a review by Ting et al. (2016) reported that the current studies on biomarkers are limited by the need for larger sample sizes, cross-validation in different populations and ethnic groups, and time-efficient and cost-effective analytical techniques.

We have performed a PubMed search for recently published studies on serum/plasma miRNAs in patients with early or late-stage DR to discover and validate miRNAs as possible diagnostic and prognostic biomarkers of DR. In particular, we have examined to what extent the limitations indicated by Joglekar et al. (2016) and Ting et al. (2016) have been addressed in the planning, implementation, and data analyses of these studies. In addition, we have searched for studies on miRNA expression in vitreous humor of eyes with PDR and compared the findings with those on miRNA expression in serum/plasma of patients with PDR.

Diabetic Retinopathy

PDR and diabetic macular edema (DME) are the two serious vision-threatening conditions in DR. DR progresses slowly before becoming symptomatic (Zou et al., 2017). The currently available treatments for DR are applicable only at advanced stages of the disease and are associated with significant adverse effects. The only therapeutic strategy in the early stages of DR is a tight control of the risk factors for DR. Therefore, new pharmacological treatments for the early stages of the disease are needed.

DR results from abnormal retinal blood vessels that are either nonproliferative or proliferative. The blood retinal barrier comprises the retinal vasculature and the retinal pigment epithelium. Endothelial cells are responsible for maintaining the blood retinal barrier, and their impairment causes increased vascular permeability (Ciulla et al., 2003). Exposure to high glucose and the resultant damage to the blood retinal barrier lead to the leakage of fluids and lipids into the retina and contribute to DR progression. DR eventually causes increasing hypoxia which stimulates abnormal neovascularization in the retina.

Various stages have been described in the progression of DR (Curtis et al., 2009; Cheung et al., 2010; NIH National Eye Institute, 2015; Solomon et al., 2017). Thus, stage 1 is mild NPDR, in which small areas of balloon-like swelling occur in the tiny blood vessels of the retina called microaneurysms, and which may leak fluid into the retina; stage 2 is moderate NPDR, as the disease progresses, blood vessels that nourish the retina may swell and distort, and may also lose their ability to transport blood. Both cause characteristic changes to the appearance of the retina and may contribute to DME; stage 3 is severe NPDR, many more blood vessels are blocked, depriving the blood supply to areas of the retina. These areas secrete growth factors that induce the retina to form new blood vessels; stage 4 is PDR, growth factors secreted by the retina stimulate the proliferation of new blood vessels which grow along the inside surface of the retina and into the vitreous humor, a gel-like fluid that fills the eye. These new blood vessels are fragile and more likely to leak and bleed. Accompanying scar tissue can contract and cause retinal detachment which can lead to permanent vision loss (NIH National Eye Institute, 2015). In an alternative classification of the stages, stage 1 is background DR (BDR) and comprises both mild and moderate NPDR, stage 2 is pre-PDR and corresponds to severe NPDR, while stage 3 is PDR (National Health Service UK, 2018). Vascular endothelial growth factor (VEGF) was shown to have a circumstantial role in the progression of DR (Boulton et al., 1998), but other growth factors such as insulin-like growth factor-1, angiopoetin-1 and -2, stromal-derived factor-1, fibroblast growth factor-2, and tumor necrosis factor may also be involved (Grant et al., 2004).

DME defines a swelling caused by the build-up of fluid (edema) in an area of the retina called the macula and is the

most common cause of vision loss among individuals with DR, with ~50% of DR subjects developing DME. DME can form at any stage of the disease, although it is more likely as DR progresses (NIH National Eye Institute, 2015).

MicroRNA Expression in Diabetic Retinopathy

A total of 15 research articles were found in the PubMed search of blood microRNAs of which nine had measured miRNAs in serum, 4 had used plasma, 1 had used extracelular vesicles extracted from serum and plasma, and 1 had used early-outgrowth endothelial progenitor cells obtained from the *in vitro* culture of peripheral blood mononuclear cells (**Table 1**). Ten of these studies had used a validation method which was RT-qPCR, while in five studies no validation method was reported. While most studies had used quite large cohorts of DR and noDR patients (≥ 40/group), there were a few that had used much smaller cohorts (< 30/group). All of the studies except one had indicated exclusion criteria, and all except three had reported gender composition of the cohorts, which in general had similar numbers of male and female subjects. The mean duration of diabetes ranged from 2 to 28 years. Only a few studies reported on medications being used by the patients, which is a serious omission as the medications may constitute confounding factors. Only eight of the studies had used receiver operating characteristics (ROC) analysis with area under curve (AUC) values to establish which microRNAs are good or fair tests to distinguish DR from noDR. Many of the studies were performed with hospital patients in China, which is a country with a high prevalence of DR (Liu et al., 2017).

In addition, three research articles were found in a PubMed search on microRNAs in vitreous humor of patients with PDR (**Table 2**). Only one of these studies had used large cohorts of patients with PDR or macular hole (MH) (29 and 30, respectively). The other two studies were made with very small numbers of patients (3 or 4/group or 7 or 10/group). All of the studies had indicated exclusion criteria and, when reported, the gender composition of the PDR and MH groups was similar. None of the studies had carried out ROC analysis and measurement of AUC values.

Blood serum

A large-scale study by Liu et al. (2018) that included 40 T1D patients with DR and 40 T1DM patients without DR (NDR) showed that using RT-qPCR validation that miR-18b, miR-19b and miR-211 in DR were significantly upregulated compared to NDR. The AUC values with 95% CI calculated by ROC curves of miR-18b, miR-19b and miR-211 were 0.779, 0.744 and 0.864, respectively, with significant diagnostic accuracy for DR, in which miR-211 is the most powerful.

A validation study by Liang et al. (2018) with 29 T2DM patients with DR and 50 T2DM patients with noDR revealed that let-7a-5p, miR-novel-chr5_15976, miR-28-3p were all significantly upregulated, while miR-151a-5p, and miR-148a-3p were all significantly downregulated between T2DM-DR and T2DM-noDR, and between early-stage T2DM-DR and T2DM-noDR. A combination of 3 miRNAs let-7a-5p, miR-28-3p, and miR-novel-chr5_15976 had an AUC value of 0.937 (sensitivity 0.923, specificity 0.950) for distinguishing T2DM-DR from T2DM-noDR, and AUC value of 0.901 (sensitivity 0.875, specificity 0.927) for distinguishing early-stage T2DM-DR from T2DM-noDR. Qin et al. (2017) examined the expression level of miR-126 in 42 DM patients with NPDR, 39 DM patients with PDR and 44 DM patients with no DR. The relative expression of miR-126 was not significantly different between NDR and NPDR groups; therefore, these two groups were combined. The level of expression of miR-126 in the PDR group was significantly lower than that in the combined NDR and NPDR group. By ROC analysis, miR-126 values differentiated PDR patients from healthy controls with an AUC of 0.976. The cut-off value was 5.02, which was associated with a sensitivity of 0.812 and a specificity of 0.903. Li et al. (2017) studied 255 patients with DR and 253 healthy controls and found by RT-qPCR that patients in DR group had significantly decreased miR-200b expression compared to healthy controls. Zampetaki et al. (2016) reported on a PREVENT-1 trial with 62 T1DM patients with DR and 64 T1DM patients without DR, and a PROTECT-1 trial with 93 T1DM patients with DR and 81 T1DM patients without DR. Penalized logistic regression analyses adjusted for age, gender, and diastolic blood pressure were performed to identify and evaluate the association of miRNAs with the incidence and progression of DR. The following miRNAs were identified in descending order of importance: miR-27b, miR-320a, miR-454, and miR-28-3p in PREVENT-1 and miR-320a, miR-122, miR-221, and miR-27b in PROTECT-1. MiR-27b and miR-320a were the two miRNAs most consistently associated with the incidence and progression of DR. Serum miR-27b was markedly lower in T1D-DR compared to T1D-noDR, while serum miR-320a was considerably higher in T1D-DR compared to T1D-noDR. By ROC analysis, addition of miR-27b and miR-320a to a panel of variables associated with disease risk (*i.e.*, age, sex, duration of diabetes, diastolic blood pressure, and level of HbA1c) improved the AUC by 0.087 for PREVENT-1 ($P = 0.027$) and by 0.034 for PROTECT-1 ($P = 0.214$). A study by Rezk et al. (2016) with 19 T2DM patients with DR and 14 T2DM patients without DR found by RT-PCR a significant decrease in miR-126 expression in T2DM-DR compared to T2DM-noDR. Qing et al. (2014) included 90 DM patients with NPDR and 90 patients with PDR, and found the expression of miR-21, miR-181c and miR-1179 was significantly higher in PDR than in NPDR. ROC analysis to assess the diagnostic sensitivity and specificity of the 3 miRNA signature for PDR gave AUC values of 0.83, 0.80, 0.87 and 0.89 in validation sets. A study by Rovira-Llopis et al. (2018) showed by RT-qPCR that levels of miR-31 in 13 T2DM patients with DR (2 with PDR) were similar to those of 18 T2DM patients without DR. Ma et al. (2017) found on RT-qPCR validation that levels of miR-3939 and miR-1910-3p were not statistically different between 45 T2DM patients with DR and 45 T2DM patients without DR.

Table 1 MicroRNAs in blood serum, plasma, EVs and EPCs of human patients with DR

Authors country	Number of patients, gender, ages	Comparison	Changes in miRNAs in DR patients	Functional outcomes	Conclusion
Blood serum					
Rovira-Llopis et al. (2018), Spain	13 T2DM patients, 10M/3F, 61.7 ± 6.5 years, diabetes duration 15.0 ± 5.5 years, with DR (2 with PDR). Patients underwent funduscopic examination. Exclusion criteria were autoimmune infections, hematological, malignant, organic or inflammatory diseases; morbid obesity (BMI ≥ 40kg/m^2); history of cardiovascular disease (including ischemic heart disease, peripheral vascular disease, stroke, and chronic disease related to cardiovascular risk); fever; intense physical exercise. After fasting overnight, venous blood was collected, centrifuged (1500 × *g*, 10 min, 4°C) and serum stored at –80°C.	18 T2DM patients, 12M/6F, 56.9 ± 8.7 years, diabetes duration 9.0 ± 4.6 years, without DR. 24 healthy subjects.	By RT-qPCR, levels of serum miR-31 in T2DM with DR patients were similar to those of T2DM noDR.		Serum miR-31 was not found to be a marker for DR.
Liu et al. (2018), China	40 type 1 diabetes (T1D) patients with DR 22M/18F, 37.7 ± 4.9 years, diabetes duration 7.0 ± 4.1 years. Exclusion criteria were cardio-cerebrovascular events, hepatic insufficiency, renal impairment, infectious diseases, pregnancy and postpartum in the previous 3 months, other severe systemic diseases. All subjects underwent ophthalmologic examination including visual function, ocular anterior and posterior segment. Serum was collected from peripheral venous blood followed by further centrifugation, and stored at –80°C until further analysis.	40 T1D patients without DR (NDR) 25M/15F, 38.9 ± 4.1 years, diabetes duration 6.1 ± 2.8 years; 40 healthy control (HC) participants 27M/13F, 36.9 ± 4.9 years.	There were no significant differences in anthropometric and biochemical indexes except for disease duration, fasting blood glucose and glycated hemoglobin among the three groups. To screen a specific miRNA expression profile, serum samples from 3 cases randomly selected in each group were compared by miRNA microarray analysis. Among 292 detectable miRNAs, there were 38 miRNAs dysregulated significantly, with 24 upregulated and 14 downregulated miRNAs comparing NDR and HC; there were 45 miRNAs dysregulated significantly, with 22 upregulated and 23 downregulated miRNAs comparing DR and HC; and there were 40 miRNAs dysregulated significantly, with 24 upregulated and 16 downregulated miRNAs comparing DR and NDR. To identify candidate miRNAs, 3 sets of miRNA expression profile were synthesized by Venn diagram analysis. The results indicated that miR-18b, miR-19b and miR-211 were significantly upregulated in each set of miRNA expression profile, while miR-23a was significantly downregulated in all sets of miRNA expression profile. To validate the candidate miRNAs selected from miRNA expression profile analysis, the expression of candidate miRNAs was quantified by qRT-PCR. The results indicated that the expression of miR-18b, miR-19b and miR-211 were significantly different among the 3 groups in agreement with microarray analysis. Serum miR-18b, miR-19b and miR-211 in NDR and DR were significantly upregulated compared to HC, respectively. In addition, serum miR-18b, miR-19b and miR-211 in DR were significantly upregulated compared to NDR. However, the expression of miR-23a among the 3 groups was not in conformity with microarray analysis.	To detect the diagnostic accuracy of candidate miRNAs, the AUC value with 95% CI was calculated by ROC curves. The results indicated that the AUC values with 95% CI of serum miR-18b, miR-19b and miR-211 were 0.779, 0.744 and 0.864, respectively, with significant diagnostic accuracy for DR, in which miR-211 is the most powerful. The AUC value with 95% CI for serum miR-23a was 0.572, with no significant diagnostic accuracy for DR. To predict target gene of miR-211, genes were searched by TargetScan 7.2, and indicated that predicted SIRT1 may be the target gene of miR-211.	Serum miR-211 may be a novel biomarker with high sensitivity and specificity associated with the occurrence and progression of DR via targeting SIRT1 gene. Stage of DR not reported.
Liang et al. (2018), China	Biomarker screening phase: 3 type 2 diabetes mellitus (T2DM) patients 2M/1F with DR, 51 years (T2DM-DR). Biomarker training phase: 10 T2DM-DR 4M/6F, 58 years. Biomarker validation phase: 29 T2DM-DR 17M/12F, 58 years; 21 Early stage T2DM-DR 14M/7F, 58 years. Subjects were excluded from the study if they had T1D, abnormal BMI or glucose tolerance, pregnancy, handicapped condition, psychological conditions, use of obesity-related medicines, and other chronic diseases (hypertension, coronary disease, systemic inflammatory diseases, acute respiratory infection, or cancers). DR is classified into two stages, NPDR (including mild, moderate and severe NPDR) and PDR. Patients with NPDR were classified as early-stage DR, and cases with PDR were grouped as late-stage DR. Fasting venous blood was collected from each subject at least 12 hr postprandial.	Biomarker screening phase: 3 T2DM patients 2M/1F without DR, 58 years (T2DM-noDR); 3 normal controls 0M/3F, 45 years. Biomarker training phase: 10 T2DM-noDR 4M/6F, 52 years. Biomarker validation phase: 50 T2DM-noDR 26M/24F, 60 years.	All the patients enrolled in the study had been clinically diagnosed with T2DM. There were no significant differences in the distribution of age, gender, BMI, smoking status, drinking status, serum glucose, and insulin levels between T2DM-DR and T2DM-noDR. However, there were significant differences of fasting C-peptide and 2-hour postprandial blood C- peptide levels between these subgoups. The miRNA expression levels were considered to be significantly different between subject groups only if they met the following criteria: 1) fold-change > 2.0 or < 2.0, 2) false discovery rate (FDR) < 0.01. Accordingly, 7 miRNAs were upregulated in T2DM-DR compared with T2DM-noDR and 47 upregulated miRNAs in T2DM-DR compared with normal controls. Among these dysregulated miRNAs was hsa-miR-novel-chr5_15976 which was not listed in miR-Base. To verify the reproducibility of the results from sequencing, the expression levels of the 8 miRNAs that showed as upregulated in T2DM-DR by sequencing were determined using qRT-PCR in 11 patients with T2DM-DR and 6 healthy controls. In agreement with the sequencing results, qRT-PCR analysis showed that expression levels of hsa-miR-novel-chr18_42128, hsa-miR-126-5p, has-miR-24-3p, and hsa-miR-novel-chr19_43683 in patients with T2DM were significantly higher than in controls. In training phase: qRT-PCR was used to compare expression levels of 21 selected miRNAs in serum samples of 10 patients with T2DM-DR and 10 patients with T2DM-noDR. Among the 21 miRNAs tested, 10 miRNAs namely hsa-let-7a-5p, hsa-miR-novel-chr5_15976, hsa-miR-28-3p, hsa-miR-151a-5p, hsa-miR-148a-3p, hsa-miR-20a-5p, hsa-miR-223-3p, hsa-miR- 92a-3p, hsa-miR-423-5p, and hsa-miR-483-5p, displayed significant differences between T2DM-DR and T2DM-noDR with FC>1.5. In validation phase: To confirm the expression differences of the 10 miRNAs selected in the training phase together with miR-122-5p, the expression levels of these miRNAs were measured in additional serum samples from 29 T2DM-DR and 50 T2DM-noDR patients. The results revealed that hsa-let-7a-5p, hsa-miR-novel-chr5_15976, hsa-miR-28-3p (all upregulated), hsa-miR-151a-5p, and hsa-miR-148a-3p (all downregulated) were significantly differentially expressed between T2DM-DR and T2DM-noDR, between early-stage T2DM-DR and T2DM-no-DR, respectively.	ROC curve was used to evaluate the diagnostic performance of differentially expressed circulating miRNAs in T2DM-DR patients. The AUC was calculated from the ROC curve for individual serum miRNA and the results showed that each miRNA had an AUC value < 0.80 to differentiate T2DM-DR or early-stage T2DM-DR from T2DM-noDR. A combination of 3 miRNAs: hsa-let-7a- 5p, miR-28-3p, and miR-novel-chr5_15976 had a markedly higher AUC value of 0.937 (sensitivity 0.923, specificity 0.950) for distinguishing T2DM-DR from T2DM-noDR, and AUC value of 0.901 (sensitivity 0.875, specificity 0.927) for distinguishing early-stage T2DM-DR from T2DM-noDR. GO analysis of T2DM-DR-associated miRNA-targeted genes showed that these T2DM-DR-associated miRNAs coexpressed with genes enriched in metabolism and inflammation-related genes. KEGG analysis suggested that T2DM-DR-associated miRNAs coexpressed with genes mainly in diabetes-related pathways, including TGF-signaling, MAPK signaling, Ras signaling, and focal adhesion. The coexpression of angiogenesis-associated genes with T2DM-DR-associated miRNAs was investigated and showed that one miRNA may correlate with multiple angiogenic genes.	The 3 miRNA signature let-7a-5p, miR-28-3p, and miR-novel-chr5_15976 from serum may serve as a diagnostic biomarker for DR and early-stage DR.

Table 1 Continued

Authors country	Number of patients, gender, ages	Comparison	Changes in miRNAs in DR patients	Functional outcomes	Conclusion
Qin et al. (2017), China	42 DM patients 23M/19F, 64.5 ± 15.0 years, 17 ≤ 60 years and 25 > 60 years, diabetes duration 19 ≤ 5 years and 23 > 5 years, with NPDR; 39 DM patients 18M/21F, 65.4 ± 16.6 years, 16 ≤ 60 years and 23 > 60 years, diabetes duration 14 ≤ 5 years and 25 > 5 years, with PDR. Patients with myocardial infarction, coronary artery bypass surgery, peripheral vascular disease, liver or renal dysfunction, or cancer were excluded. Fundus fluorescein angiography (FFA) was made to determine the condition of fundus in DM patients. Peripheral blood samples were collected after 12-hour fast, centrifuged at 3,000 g for 10 minutes at 8°C, and supernatants stored at –80°C. All blood samples were processed not more than 4 hour after they were collected.	44 DM patients 20M/24F, 64.9 ± 15.0 years, 19 ≤ 60 years and 25 > 60 years, diabetes duration 21 ≤ 5 years and 23 > 5 years with no DR (NDR); 59 healthy control participants 33M/26F, 65.5 ± 15.0 years, 25 ≤ 60 years and 34 > 60 years.	No significant differences in age, gender, smoking status, and other conditions were observed between DM patients and controls. Serum miR-126 levels were significantly reduced in NDR, NPDR and PDR groups compared to healthy control group. However, the relative expression of miR-126 was not significantly different between NDR and NPDR groups. Therefore, these two groups were combined into one group in further analysis. The level of miR-126 in NDR and NPDR group was 4.58 ± 2.36, which was significantly higher than that level in PDR group of 2.76 ± 1.34.	ROC curve was used to evaluate whether serum miR-126 level could be used as a potential diagnostic marker for different stages of DR. Serum miR-126 levels differentiated DM patients from healthy controls with AUC value of 0.932. The cut-off value was 8.43, which was associated with a sensitivity and a specificity of 84.75% and 93.60%, respectively. Serum miR-126 levels differentiated NDR and NPDR patients from healthy controls with an AUC of 0.919. At the cut-off value of 8.43, it had 84.75% sensitivity and 94.41% specificity. Serum miR-126 values differentiated PDR patients from healthy controls with an AUC of 0.976. The cut-off value was 5.02, which was associated with a sensitivity of 81.21% and a specificity of 90.34%.	Serum miR-126 can serve as a non-invasive biomarker for screening retinal endothelial injury and early diagnosis PDR. Study did not indicate whether patients had TD1 or T2DM.
Ma et al. (2017), China	45 T2DM patients with DR, 66.2 ± 8.4 years, diabetes duration 16.8 ± 7.4 years (DR group). All of the subjects underwent fundus fluorescein angiography. Subjects with acute or chronic inflammatory disease, type 1 diabetes, maturity-onset diabetes of the young, or mitochondrial diabetes were excluded. Peripheral blood samples were collected following a 12-hour fast and serum extracted.	45 T2DM patients without DR, 65.4 ± 8.0 years, diabetes duration 16.0 ± 9.5 years (NDR group).	The RNA samples from 5 DR cases and 5 NDR controls were analysed by microarray assay. Two miRNAs (miR-3939 and miR-1910-3p) were higher in DR patients than in NDR patients. These two serum miRNAs were validated in 45 DR cases and 45 matched NDR controls using RT-qPCR but no statistically significant difference was found..		The findings indicated that miR-3939 and miR-1910-3p may not play important roles in the development of DR. However, studies with a larger sample size are needed to confirm these findings.
Li et al. (2017), China	255 patients with DR 134M/121F, 61.5 ± 11.9 years., disease duration 8.2 ± 2.4 years. All patients were given eye examinations and a general physical examination. Exclusion criteria were no history of hepatitis, acute and chronic infection, and malignant tumor; no systemic diseases such as cardiovascular and cerebrovascular diseases, inflammatory diseases, tissue proliferative diseases, autoimmune diseases and no other eye infections and eye diseases. After fasting 12–14 hours, peripheral venous blood was collected, centrifuged at 1,500rpm for 15 minutes, and serum isolated.	253 healthy controls 140M/113F, 60.2 ± 7.7 years. All subjects were examined by fundus photography and fundus fluorescein angiography. Exclusion criteria were no retinopathy and other eye diseases such as age-related macular diseases and ischemic optic neuropathy; no family history of glaucoma, ocular trauma, and other eye diseases.	There were no significant differences in gender and age between the DR group and control group. Patients in DR group had higher BMI, total cholesterol, triglycerides, HDL, glycosylated hemoglobin, blood glucose and blood pressure compared to healthy controls. By RT-qPCR, patients in DR group had significantly decreased serum miR-200b expression and significantly increased serum VEGFA mRNA expression compared to healthy controls. Correlation analysis showed that miR-200b was negatively correlated with VEGFA in both groups.	VEGFA was confirmed as a target gene of miR-200b.	MiR-200b might alleviate DR development by downregulating its target gene VEGFA. Study did not indicate numbers of patients with TD1 and T2DM.
Zampetaki et al. (2016), several countries including UK, USA	PREVENT-1 trial: 62 T1D patients 33M/29F, 31.1 ± 8.1 years, diabetes duration 7.3 ± 3.8 years, with DR. PROTECT-1 trial: 93 T1D patients 58M/35F, 31.5 ± 9.1 years, diabetes duration 10.9 ± 4.0 years, with DR. Serum samples were collected.	PREVENT-1 trial: 64 T1D patients 37M/27F, 27.7 ± 8.1 years, diabetes duration 7.3 ± 3.6 years, without DR. PROTECT-1 trial: 81 T1D patients 42/39F, 31.0 ± 8.5 years, diabetes duration 10.6 ± 4.0 years, without DR.	Penalized logistic regression analyses adjusted for age, gender, and diastolic blood pressure were performed to identify and evaluate the association of miRNAs with incidence and progression of DR. The following miRNAs were identified in descending order of importance: miR-27b, miR-320a, miR-454, and miR-28-3p in PREVENT-1 and miR-320a, miR-122, miR-221, and miR- 27b in PROTECT-1. Since combinations of miRNAs can be superior to individual miRNAs (Zampetaki et al., 2010; 2012a; 2012b), "traditional" logistic regression was performed for the two miRNAs most consistently associated with incidence and progression of DR: miR-27b and miR-320a. Serum miR-27b was markedly lower in T1D-DR compared to T1D-noDR, while serum miR-320a was considerably higher in T1D-DR compared to T1D-noDR.	By ROC analysis, addition of information on miR-27b and miR-320a to a model of a panel of variables associated with disease risk (*i.e.*, age, sex, duration of diabetes, diastolic blood pressure, and level of HbA1c) improved the AUC by 0.087 for PREVENT-1 ($P = 0.027$) and by 0.034 for PROTECT-1 ($P = 0.214$). Proteomics analyses in ECs showed thrombospondin-1 as a common target for both miR-320a and miR-27b.	Two angiogenic miRNAs, miR-320a and miR-27b are potential biomarkers for DR.
Rezk et al. (2016), Egypt	19 T2DM patients 10M/9F, 50.1 ± 10.3 years, diabetes duration 6.1 ± 1.1 years, with DR. Exclusion criteria were liver cirrhosis, malignancy, infection, inflammation, bronchial asthma, or heart failure. Serum and plasma were obtained from blood collected after 8-hour and 12-hour fast and stored at –20°C.	14 T2DM patients 6M/8F, 45.2 ± 7.3 years, diabetes duration 1.7 ± 0.5 years without DR or other complications.	By RT-PCR, a significant decrease in serum miR-126 was found in T2DM-DR compared to T2DM-noDR.	MiR-126 is highly abundant in endothelial cells and has an important role in maintaining endothelial homeostasis, vascular integrity and regulating angiogenesis (Wang et al., 2008). It assists VEGF signaling by suppressing two negative regulators of the VEGF pathway, Sprouty-related protein (SPRED 1) and phosphoinositol-3 kinase regulatory subunit 2 (PIK3R2/p85-β) (Fish et al., 2008).	Serum miR-126 expression could be a good marker for DR.

Table 1 Continued

Authors country	Number of patients, gender, ages	Comparison	Changes in miRNAs in DR patients	Functional outcomes	Conclusion
Qing et al. (2014), China	90 DM patients 45M/45F, 46 < 60 years, 44 ≥ 60 years, diabetes duration 17 < 5 years, 73 ≥ 5 years, with NPDR. 90 DM patients 48M/42F, 55 < 60 years, 35 ≥ 60 years, diabetes duration 16 < 5 years, 74 ≥ 5 years, with PDR. Patients underwent ophthalmologic examination including visual acuity, slit lamp examination, fundus contact lens examination and fluorescein angiography. Blood was collected from fasting participants and serum obtained.	20 Controls 10M/10F, 10 < 60 years, 10 ≥ 60 years.	An initial screening of miRNA expression was performed through TaqMan Low Density Array (TLDA). The candidate miRNAs were validated by individual reverse transcription quantitative real-time PCR (RT-qPCR) arranged in an initial and a two-stage validation sets. Moreover, additional double-blind testing was performed in 20 patients clinically suspected of having DR to evaluate the diagnostic value and accuracy of the serum miRNA profiling system in predicting PDR. Upregulated expression of 18 miRNAs and downregulated expression of 1 miRNA was found by miRNA expression microarray of serum of NPDR and PDR patients. The 18 candidate miRNAs were examined by RT-qPCR in a training sample set (30 PDR and 30 NPDR). All 18 upregulated miRNAs were detected and the downregulated miRNA indicated no difference between PDR and NPDR. The difference in miR-21, miR-181c and miR-1179 expression level between PDR and NPDR was statistically significant which was consistent with the results of RT-PCR-based TLDA. To validate the accuracy and specificity of these miRNAs as a PDR potential signature, their expression levels were examined through internal and external samples. The expression levels of the 3 miRNAs in serum of PDR were all significantly higher than those in NPDR.	ROC curve analysis to assess the diagnostic sensitivity and specificity of the 3 miRNA signature for PDR gave AUC 0.83 (95% *CI*, 0.79–1.00), 0.80 (95% *CI*, 0.81–0.98), 0.87 (95% *CI*, 0.86–0.99) and 0.89 (95% *CI*, 0.86–0.96) for serum samples in validation sets. These results indicated that the 3 serum miRNA signature can serve as a novel non-invasive approach for the early screening of PDR from NPDR.	The 3 serum microRNAs can serve as a monitoring factor for detecting the progression of PDR from NPDR. The numbers of T2DM and T1D patients in the NPDR and PDR groups was not reported.
Blood plasma					
Dantas da Costa e Silva et al. (2018), Brazil	46 T2DM patients 22M/24F, 60.0 ± 9.2 years, 14 non-white/32 white, diabetes duration 14.5 ± 7.2 years, with NPDR. 49 T2DM patients 30M/19F, 63.8 ± 6.6 years, 16 non-white/33 white, diabetes duration 19.7 ± 8.4 years, with PDR. Exclusion criteria included any clinical condition that impairs fundus examination such as severe cataract. Peripheral blood samples were collected, centrifuged 2,500 × *g* for 15 minutes at 4°C. Plasma was stored at –70°C.	91 T2DM patients 30M/61F, 60.3 ± 8.3 years, 23 non-white/68 white, diabetes duration 14.8 ± 7.7 years, without DR. 20 healthy controls 9M/11F, 6 non-white/14 white, 45.5 ± 7.5 years with no known personal and/or first-degree history of diabetes.	By RT-qPCR, T2DM- noDR patients had approximately 2-fold lower plasma levels of miR-200b compared to healthy controls, while the levels of miR-29b were not significantly different between them. The mean levels of miR-29b were 40% lower in PDR patients compared to those without DR. The same trend was observed for miR-200b, but the difference between the three groups of diabetic patients did not reach significance. Using logistic regression, plasma miR-29b was associated with PDR, but plasma miR-200b was not associated with PDR. However, miR-29b levels did not remain associated with PDR after adjusting for the demographic and clinical variables that were also associated with this outcome in the univariate analyses.		PDR was inversely associated with plasma levels of miR-29b and miR-200b. However, these associations were lost after controlling for demographic and clinical covariates.
Zou et al. (2017), China	75 T2DM patients 41M/34F, 48.3 ± 8.6 years, diabetes duration 9.3 ± 2.8 years with DR (DR group). Patients underwent routine fundus examination and fundus fluorescence angiography examination. Exclusion criteria included acute complications like diabetic ketosis, hyperglycemic coma, severe stress such as recent cardiovascular events, trauma operation, acute or chronic infection, hepatic disease, and other endocrine metabolic disease. All subjects fasted 8–12 hours and venous blood collected. Blood samples centrifuged at 3,000rpm for 10 minutes at room temperature to obtain upper plasma that was stored at –80°C.	65 T2DM patients without DR 36M/29F, 49.3 ± 8.5 years, diabetes duration 7.6 ± 2.8 years (NDR group); 127 healthy subjects 66M/61F, 47.3 ± 9.8 years, none were associated with a history or family history of T2DM or other eye disease (control group).	Compared with the control group, the course of disease was lengthened and the levels of FBG, HbA1c, TC, LDL- C, FPG, TG, BUN, Fins, Cr, IL-1, IL-6, TNF-α and VEGF were increased, but the HDL-C level was decreased in the DR and NDR groups. The course of disease was longer and the levels of FBG, HbA1c, FPG, IL-1, IL-6, and VEGF in the DR group were significantly higher than those in the NDR group. There were no significant changes in age, gender, BMI, TC, HDL-C, LDL-C, TG, BUN, FIns, Cr among the three groups. The plasma miR-93 expression and mRNA expressions of IL-1, IL-6, TNF-α and VEGF in the DR group increased significantly compared to those in the NDR group and control group.	ROC curve showed that the best cut-off of plasma miR-93 for detection of T2DM-DR was 1.31, with AUC value 0.866 and sensitivity of 73.33% and specificity 89.24%, indicating that miR-93 expression has a diagnostic value in T2DM-DR. Plasma miR-93 expression was positively correlated with the course of disease, HbA1c, FPG, TNF-α and VEGF while no significant correlation was found between plasma miR-93 expression and gender, age, BMI, FBG, TC, HDL-C, LDL-C, TG, BUN, FIns, Cr, IL-1 and IL-6.	Plasma miR-93 is associated with the progression of T2DM-DR and can serve as a diagnostic marker for T2DM-DR.
Jiang et al. (2017), China	73 T2DM patients 32M/41F, 48.8 ± 7.1 years, diabetes duration 9.1 ± 3.1 years with non-proliferative/ background DR (BDR/NPDR group); 51 T2DM patients 28M/23F, 50.8 ± 10.2 years, diabetes duration 13.6 ± 3.8 years with proliferative DR (PDR group). Exclusion criteria included patients with diabetic ketoacidosis, diabetic hyperosmolar coma and other acute complications of diabetes; patients in severe stress such as recent cardiovascular events, trauma surgery *etc.*; patient suffering from acute or chronic infection; patients with liver disease; and patients combined with other endocrine and metabolic diseases. All subjects were fasted for 8–12 hours and venous blood sample collected, centrifuged at 3,000rpm for 10 minutes and upper plasma stored at –80°C.	65 T2DM patients 34M/31F without DR, 47.8 ± 8.1 years, duration of diabetes 4.2 ± 1.3 years (NDR group). 115 non-T2DM healthy individuals 60M/55F, 48.5 ± 7.3 years (control group).	There were no significant difference in gender, age, BMI, FBG, Cr, BUN, FINS, TC, TG and LDL-C levels among the NDR, BDR, PDR and control groups. Disease course, HbA1c, FPG levels and HOMA-IR levels were significantly increased in the NDR group compared with those in the control group. Disease course, HbA1c and FPG were significantly increased in the PDR group compared with the BDR group, showing a gradually increasing trend from the NDR group to the BDR group to the PDR group. By RT-qPCR, the plasma miR-21 expression in the NDR group was not significantly different to the control group. The expression of miR-21 was significantly increased in the BDR and PDR groups compared to NDR and control groups, and the expression in the PDR group was significantly higher than in the BDR group. The miR-21 expression in each group was positively correlated with disease course, HOMA-IR, HbA1c and FPG. However, there was no significant correlation between miR-21 and TG, TC, Cr, BUN, HDL-C, FINS or LDL-C.	ROC curve analysis gave an AUC value of 0.825,with a sensitivity of 66.1% and a specificity of 90.4%, for plasma miR-21 in diagnosing DR, which indicated that plasma miR-21 expression had a relative predictive value for T2DM-DR. ROC curve analysis to measure the diagnostic value of miR-21 in PDR gave an AUC of 0.830, with a sensitivity of 72.5% and a specificity of 79.5%, indicating that plasma miR-21 expression had a relative predictive value for PDR.	Plasma nmiR-21 expression was increased in the development of T2DM-DR and PDR, and can be used as an indicator for the severity of T2DM with DR.

Table 1 Continued

Authors country	Number of patients, gender, ages	Comparison	Changes in miRNAs in DR patients	Functional outcomes	Conclusion
Yang et al. (2015), China	20 T2DM patients with background DR (BDR group); 20 T2DM patients with PDR (PDR group). All patients examined by ophthalmoscopy and fluorescein angiography. The M/F ratio of T2DM patients was 1:1, mean age 52 years. Exclusion criteria were heart, liver or kidney disease history, other allergic history, corticosteroid or any other immunosuppressive agents. Venous blood was collected. Peripheral blood mononuclear cells (PBMC) were obtained by use of lymphocyte separation solution and centrifugation 2,000rpm for 20 minutes. Then CD4+CD25+Foxp3+ T cells (Treg cells) were isolated from PBMC cells. The TGF-β level in the plasma was measured.	20 T2DM patients with no DR (NDR group). 20 healthy subjects 10M/10F with no diabetes or ophthalmic disease history, mean age 50 years were normal controls (NC group).	The percentages of Treg cells in the peripheral blood of patients in BDR, PDR and NDR groups were significantly decreased compared to NC group. The percentages of Treg cells in BDR and PDR groups were significantly lower than in NDR group, and percentage of Treg cells in PDR group was significantly lower than in BDR group. The expression level of miR-155 in the peripheral blood of patients in BDR, PDR and NDR groups was significantly increased compared to NC group. The expression level of miR-155 in BDR and PDR groups was significantly higher than in NDR group, and expression level of miR-155 in PDR group was significantly higher than in BDR group. The expression level of TGF-β in the peripheral blood of patients in BDR and PDR groups was significantly increased compared to NDR and NC groups. The expression of miR-155 was negatively related to the percentages of Treg cells.		MiR-155 may play an important role in the pathogenesis of T2DM-DR by regulating the percentage of Treg cells and TGF-β expression in peripheral blood.
Blood extracellular vesicles (EVs)					
Mazzeo et al. (2018), Italy	7 type 1 diabetes (T1D) subjects 4M/3F, 39.3 ± 5.9 years, diabetes duration 28.0 ± 12.8 years, with PDR (DR group). Exclusion criteria: other diabetic complications, systemic diseases limiting life expectancy (*e.g.*, cancer, cirrhosis) or other autoimmune diseases. All diabetic patients were on multiple daily insulin injections. Overnight fasting venous blood was collected for plasma separation and clot activator for serum. EVs were collected from serum and plasma by centrifugation 3,000 × *g* for 30 minutes to remove debris, apoptotic bodies and platelets, followed by ultracentrifugation 100,000 × *g* for 3 hours at 4°C of the cell-free supernatants.	7 diabetic subjects 4M/3F, 46.1 ± 11.7 years, diabetes duration 27.3 ± 14.2 years, without retinopathy (noDR group); all diabetic patients were on multiple daily insulin injections. 7 healthy controls 4M/3F, 41.0 ± 10.6 years (CTR group).	EVs showed similar mean size among the 3 groups (DR, noDR, CTR) and between serum and plasma. The number of EV/ml was 2.5-fold higher in both diabetic groups compared to healthy controls, but within each group there was no difference between serum and plasma. Among diabetic patients, there was correlation between HbA1c levels and EV concentration in serum or plasma. Microarray analysis revealed 11 miRNAs to be differentially expressed in the 3 groups, especially in regard to DR group compared with CTR. In particular, 6 miRNAs were upregulated in DR vs. CTR: miR-17-5p, miR-21-3p, miR-30b-5p, miR-106a, miR-139-5p, and miR-484. Among these, miR-21-3p was significantly upregulated also in noDR vs. CTR, and miR-30b-5p in DR vs. noDR. Expression of the differentially regulated miRNAs was checked by qRT-PCR and 3 out of 11 were confirmed to change significantly among groups. In particular, miR-150-5p was strongly decreased in DR group compared to noDR and CTR, while miR-21-3p was 5-fold upregulated in DR and 3-fold upregulated in noDR compared to CTR. In addition, miR-30b-5p was 5-fold increased in DR group compared to noDR and CTR, but was unchanged in noDR group. Expression of miR-150-5p, miR-21-3p, or miR-30b-5p did not correlate with HbA1c levels in diabetic subjects.	*In vitro* experiments showed that EVs from plasma of DR subjects induced detachment of human retinal pericytes (HRP) and migration of HRP and human microvascular endothelial cells (HMEC), increased the permeability of EC/HRP bilayers and formation of vessel-like structures, when compared to EVs from CTR subjects.	EVs extracted from plasma of DR subjects were able to induce features of retinopathy in *in vitro* models of retinal microvasculature. miR-150-5p, miR-21-3p and miR-30b-5p may serve as potential biomarkers for PDR.
Peripheral blood endothelial progenitor cells (EPCs)					
Garcia de la Torre et al. (2015), Spain	41 T1D patients, diabetes duration 30.3 ± 10.3 years, with DR (DR group). Exclusion criteria were pregnancy, hepatic insufficiency, renal insufficiency, overt macrovascular disease (ischemic heart disease, cerebrovascular disease, peripheral vascular disease) in the previous 6 months, or other severe diseases. Venous blood was collected after overnight fast ≥ 12 hours. PBMCs were isolated and cultured in EGM-2MV containing VEGF, FGF-2, EGF, IGF, ascorbic acid, hydrocortisone, gentamycin with 10% FCS to obtainEPCs. Identification of EPCs was performed after 7 days of culture by dual positive staining for Dil-Ac-LDL and lectin.	35 T1D patients, diabetes duration 16.9 ± 10.8 years, without DR (noDR group). 38 healthy subjects (control group).	T1D patients with DR had a longer duration of the disease than those without DR, presented with other diabetic complications more frequently, smoked more, and received statin and ACE inhibitor/ARB treatment more often, but did not differ in the degree of metabolic control and other clinical characteristics. T1D patients with DR had a significantly increased expression of miR-221 in EPCs compared to T1D patients without DR, but there was no difference among different degrees of DR progression. Healthy controls had a similar miR-221 expression to T1D patients without DR, but lower than that in T1D patients with DR. Data obtained in this study showed that miR-221 expression was increased in patients treated with ACE inhibitors or angiotensin II receptor blocker (ARB) compared with those without treatment.	ROC analysis for miR-221 expression in early-outgrowth EPCs gave an AUC value of 0.696. Considering miR-221 expression as a marker for DR, a cut-off point of 1.14 would have a sensitivity of 0.72 and specificity of 0.60.	MiR-221 may be useful for monitoring DR progression and potential therapeutic target.

EVs: Extracelular vesicles; EPCs: endothelial progenitor cells; DR: diabetic retinopathy; T2DM: type 2 diabetes mellitus; T1DM: type 1 diabetes mellitus; NPDR: nonproliferative diabetic retinopathy; PDR: proliferative diabetic retinopathy; ROC: receiver operating characteristics; AUC:area under curve; BMI: body mass index; VEGF: vascular endothelial growth factor; FBG: fasting blood glucose; HbA1c: glycosylated hemoglobin; TC: total cholesterol; LDL-C: low-density lipoprotein cholesterol; FPG: fasting plasma glucose; TG: triglyceride; BUN: blood urea nitrogen; Cr: creatinine; IL: interleukin; HDL-C: high-density lipoprotein cholesterol; TNF: tumor necrosis factor; FINS: fasting insulin.

Table 2 MicroRNAs in vitreous humor of human patients with DR

Authors country	Number of patients, gender, ages	Comparison	Changes in miRNAs in DR patients	Functional outcomes	Conclusion
Vitreous humor					
Gomaa et al. (2017), Egypt	29 eyes of 29 patients 17 TD1/12 T2DM, 40–71 years, diabetes duration 8–30 years, with PDR which were indicated for pars plana vitreotomy (PPV) due to long-standing (> 3 months) vitreous or preretinal hemorrhage and/or tractional retinal detachment affecting or threatening the macula. Exclusion criteria included patients with other systemic diseases, panretinal photocoagulation, or intravitreal anti-VEGF antibody injection in the last 6 months, previous PPV, presence of rubeosis iridis, neovascular glaucoma, rhegmatogenous retinal detachment, retinal vascular occlusion, or a history of intraocular inflammation. Patients subjected to a full ophthalmological evaluation. An undiluted vitreous sample was collected at the beginning of PPV, centrifuged, and stored at –80°C. Venous blood samples were collected in the morning before PPV.	30 eyes of 30 age- and gender-matched nondiabetic patients 13M/17F indicated for PPV due to idiopathic macular holes (IMH) as a control group. IMH is considered a nonproliferative and noninflammatory disorder.	There was no significant difference between the two groups regarding age or gender. By RT-qPCR, miR-200b expression in the vitreous samples was significantly higher in the PDR group than the IMH group. Using multivariate regression analysis and after adjustment for age, gender, and VEGF level, the association between miR-200b and PDR was still significant. Also VEGF was significantly higher in vitreous samples from PDR group than IMH group. No significant correlation was found between vitreous miR-200b and VEGF in the PDR group.	No significant correlation was found between miR-200b and diabetes duration or HbA1c. For VEGF expression in the vitreous, there was a significant positive correlation with diabetes duration and HbA1c. VEGF and miR-200b in the vitreous did not significantly correlate with age.	MiR-200b may be involved in the pathogenesis of PDR but through VEGF-independent mechanisms.
Usui-Ouchi et al. (2016), Japan	3 PDR with PVR (proliferative vitreoretinopathy) patients 2M/1F, 46–64 years, as PVD (proliferative vitreoretinal disease) group. *Validation*: 10 PDR with PVR patients. Exclusion criteria were systemic diseases such as autoimmune diseases, cancer, histories of ocular diseases and/or ocular surgeries. Vitreous samples were collected, centrifuged at 200 × *g* for 10 minutes, and stored at –80°C.	3 patients with macular hole (MH) 2M/1F, 54–69 years, as control group. *Validation*: 7 patients with MH.	By qPCR, approximately 46% of the miRNAs in the vitreous humor samples of the PVD group showed changes of >3-fold in expression compared with the control group. Hierarchical clustering of the fold changes between the control and PVD groups showed distinct clusters with 23 miRNAs consistently upregulated in the PVD group and 12 miRNAs consistently downregulated. By performing a volcano plot filtering against these miRNA expression profiles, with a change in miRNA expression considered statistically significant if the fold change was > 2 and *P* value < 0.05, the expression levels of 5 miRNAs (let-7c, miR-204, miR-216b, miR-9, miR-139-5p) were significantly downregulated in the PVD group, and the expression levels of 9 miRNAs (miR-16, miR-92a, miR-130b, miR-21, miR-320, miR-106b, miR-423-5p, miR-210, miR-532-3p) were significantly upregulated in the PVD group. Expression of 3 microRNAs miR-21, miR-204 and let-7c in vitreous humor of 7 patients with MH and 10 PDR with PVR patients was examined by RT-PCR. The expression level of miR-21 in the vitreous humor of PDR patients was significantly higher than that of MH patients, and the expression levels of miR-204 and let-7c in the vitreous humor of PDR patients were significantly lower than in the MH group.		MiR-21 is a potential disease-modifying microRNA in the vitreous humor that is involved in the development of retinal fibrosis and may be a marker of PVD.
Hirota et al. (2015), Japan	4 patients 2M/2F, 46–67 years, with PDR who underwent vitreotomy. Exclusion criteria were other systemic diseases, history of ocular diseases and ocular surgeries. Peripheral blood was collected from the patients before surgery, centrifuged 3,500rpm for 7 minutes to isolate the blood serum. Vitreous was collected, centrifuged 400 × *g* (1,500rpm) for 10 minutes. All samples stored at –80°C.	4 patients 2M/2F, 57–75 years. with IMH. Exclusion criteria were systemic diseases such as hypertension, DM, autoimmune diseases, and cancer, history of ocular diseases and ocular surgeries.	By RT-qPCR, 33 miRNAs were expressed in vitreous of four IMH patients and 51 miRNAs were commonly expressed in vitreous of four PDR patients. 26 miRNAs were expressed in both IMH and PDR eyes. Among these, the expression levels of 20 miRNAs were not significantly different between the two groups. However, 6 miRNAs, hsa-miR-15a, hsa-miR-320a, hsa-miR-320b, hsa-miR-93, hsa-miR-29a, and hsa-miR-423-5p, were expressed significantly higher in the PDR eyes. Several miRNAs including hsa-miR-21, hsa-miR-125b, and hsa-miR-210 were highly expressed (over 2-fold) in the vitreous compared to the serum in both IMH patients and PDR patients. The fold change in vitreous relative to serum for expression levels of three miRNAs, hsa-miR-23a, hsa-miR-320a, and hsa-miR-320b, were significantly higher in the PDR group than in the MH group.	Of the six miRNAs expressed at higher levels in the vitreous of eyes with PDR, five miRNAs miR-15a, miR-320a, miR-320b, miR-93, and miR-29a have been reported to play important roles in angiogenesis (Wang et al., 2009; Long et al., 2010; Fang et al., 2011; Yin et al., 2012). In particular, miR-15a is known to inhibit angiogenesis by suppressing fibroblast growth factor (FGF2) and VEGF (Yin et al., 2012). In addition, recent studies have shown that miR-320 regulates tumor angiogenesis by silencing neuropilin 1 (Wu et al., 2014). It also reduces the proliferation and migration of myocardial microvascular endothelial cells by targeting insulin-like growth factor 1 in type 2 diabetic rats (Wang et al., 2009). In addition, miR-93 is reported to regulate VEGF expression in experimental models of diabetes (Long et al., 2010).	The expression of several miRNAs related to angiogenesis and fibrosis was significantly higher in vitreous of PDR eyes.

DR: Diabetic retinopathy; T2DM: type 2 diabetes mellitus; T1DM: type 1 diabetes mellitus; NPDR: nonproliferative diabetic retinopathy; PDR: proliferative diabetic retinopathy; VEGF: vascular endothelial growth factor; HbA1c: glycosylated hemoglobin.

Blood plasma

Dantas de Costa e Silva et al. (2019) performed a large-scale study that included 46 T2DM patients with NPDR, 49 T2DM patients with PDR, and 91 T2DM patients without DR. This study was carried out in Brazil and most of the patients recruited were white. By RT-qPCR, the levels of miR-29b were significantly lower in PDR patients compared to those without DR. The same trend was observed for miR-200b, but the difference was not significant. Using logistic regression, miR-29b was associated with PDR, but miR-200b was not. However, miR-29b levels did not remain associated with PDR after adjusting for the demographic and clinical variables that were also associated with this outcome in the univariate analyses. A study by Zou et al. (2017) with 75 T2DM patients with DR and 65 T2DM patients without DR found using RT-qPCR that miR-93 was significantly increased in the DR group compare to the noDR group. ROC analysis showed that the best cut-off of miR-93 for detection of T2DM-DR was 1.31, with AUC value 0.866 (sensitivity 73.33%, specificity 89.24%, indicating that miR-93 expression has diagnostic potential for T2DM-DR. Another large-scale study reported by Jiang et al. (2017) recruited 73 T2DM patients with BDR/NPDR, 51 T2DM patients with PDR, and 65 T2DM patients without DR. By RT-qPCR, the expression of miR-21 was significantly increased in the BDR and PDR groups compared to noDR, and the expression in the PDR group was significantly greater than in the BDR group. ROC analysis gave an AUC value of 0.825 (sensitivity 66.1%, specificity 90.4%) for miR-21 in diagnosing DR, which indicated that miR-21 expression had a relative predictive value for T2DM-DR. ROC curve analysis to measure the diagnostic value of miR-21 in PDR gave an AUC of 0.830 (sensitivity 72.5%, specificity 79.5%) indicating that miR-21 expression had a relative predictive value for PDR. MiR-21 can be used as an indicator for the severity of T2DM DR. Yang et al. (2015) examined miR-155 expression in 20 T2DM patients with BDR, 20 T2DM patients with PDR, and 20 T2DM patients without DR. By RT-PCR, the level of miR-155 in the BDR and PDR groups was significantly greater than in the noDR group, and the level of miR-155 in the PDR group was significantly greater than in the BDR group. Thus, miR-155 can serve to monitor the progression of DR.

Peripheral blood extracellular vesicles

Mazzeo et al. (2018) collected extracellular vesicles from serum and plasma of seven T1DM patients with PDR and seven diabetic patients without DR. By RT-qPCR validation, the expression of miR-150-5p was significantly lower in the DR group compared to noDR group, while the expression of miR-30b-5p was significantly greater in the DR group compared to noDR group.

Peripheral blood endothelial progenitor cells

Garcia de la Torre et al. (2015) examined miR-221 expression in early-outgrowth endothelial progenitor cells obtained from peripheral blood mononuclear cells of 41 T1DM patients with DR and 35 T1DM patients without DR. By RT-PCR, the expression of miR-221 was significantly greater in the DR group compared to noDR group, but there was no difference among different degrees of DR progression. ROC analysis for miR-221 expression gave an AUC value of 0.696, and with a cut-off point of 1.14 had a sensitivity of 0.72 and specificity of 0.60 as a marker for DR.

Vitreous humor

Gomaa et al. (2017) performed a large-scale study with 29 eyes of 29 patients (17 T1DM, 12 T2DM) with PDR and 30 eyes of 30 nondiabetic patients with MH as a control group. By RT-qPCR, miR-200b expression was significantly greater in the PDR group than the MH group. Using multivariate regression analysis and after adjustment for age, gender, and VEGF level, the association between miR-200b and PDR was still significant. Usui-Ouchi et al. (2016) in a validation study with 10 PVD patients (PDR with PVR) and 7 patients with MH found using RT-PCR that the expression level of miR-21 in PDR patients was significantly greater than in MH patients, and the expression levels of miR-204 and let-7c in PDR patients were significantly lower than in MH patients. In addition, a small-scale study by Hirota et al. (2015) with four patients with PDR and four patients with MH observed by RT-qPCR that the expression levels of miR-15a, miR-320a, miR-320b, miR-93, miR-29a, and miR-423-5p, were significantly greater in the PDR eyes than in MH eyes. The fold changes in vitreous relative to serum for expression levels of three miRNAs, miR-23a, miR-320a, and miR-320b, were significantly greater in the PDR group than in the MH group.

Future Perspectives

DR is the leading cause of new cases of blindness and impaired vision in adults aged 20–65 years (Sokol-McKay). The basic pathological change of DR is retinal neovascularization and fibrosis hyperplasia. Recent studies have indicated that DR is an inflammatory disease, with oxidative stress, formation of advanced glycation end-products and increased expression of VEGF all contributing to the inflammatory response (Semeraro et al., 2015; Rubsam et al., 2018). The treatments for PDR are panretinal laser photocoagulation and vitrectomy in selected cases, and anti-VEGF injections have recently been used as another treatment option. However, serious systemic side-effects can develop and there may be reoccurrence of the neovascularization a few months after the anti-VEGF injection (Salam et al., 2011). There is an urgent need to develop new treatments for the management of PDR.

At present DR can only be diagnosed *via* formal examination of the eye by a trained specialist. Studies have shown that circulating miRNAs play a significant role in the development of diabetes and they may be a more sensitive way to predict development of the disease than the currently available tools (Jimenez-Lucena et al., 2018). To date, few studies have investigated the relationship between circulating miRNA levels and the development of retinopathy in diabetic patients, with most studies focussing on diabetic rat models or

retinal endothelial cells cultured in high glucose conditions. Circulating miRNA levels can be used for the early prediction of DR with high sensitivity and specificity, and altered circulating miRNA levels may provide a novel minimally invasive biomarker for the early detection of DR (Ma et al., 2017). It has been shown that microRNAs have remarkable chemical and physical stability in various body fluids.

Most of the studies reviewed showed that circulating microRNAs could distinguish diabetic patients (T1DM and T2DM) with retinopathy from healthy controls and diabetic patients without retinopathy (T1DM noDR and T2DM noDR) (**Table 1** and **Figure 1**). In addition, serum expression levels of miR-126, miR-21, miR-181c, miR-1179, and plasma expression levels of miR-21, miR-155, were dysregulated in PDR and therefore served as markers of disease and for monitoring disease progression. Interestingly, miR-21 expression was greater in vitreous humor of PDR patients than in MH patients (controls). Also, miR-320a and miR-320b had increased expression in vitreous humor in PDR than in MH, and Zampetaki et al. (2016) had identified a higher serum expression level of miR-320 in DR patients compared to patients without DR. However, several of the reviewed studies had small numbers of patients, especially those analyzing vitreous humor, and this is an important limitation of these studies. While vitreous humor cannot be collected from eyes of diabetic patients without DR due to ethical reasons, collection immediately postmortem from eyes of deceased diabetic patients with and without DR and from those of deceased healthy subjects could provide important information towards establishing biomarkers of DR, especially PDR, with high sensitivity and specificity.

The microRNA findings from screening/discovery studies need to be confirmed or discounted by validation studies with large group sizes. Very few of the validation studies reported relative fold change values for DR. Such quantitative information would emphasize miRNAs with the greatest evidence in support of their role as a biomarker of DR in humans. Currently qPCR is the favored method for determining miRNA expression due to its accuracy, simplicity, reproducibility and lower cost than other hybridization or sequencing-based systems (Git et al., 2010). Ma et al. (2017) found by microarray analysis that serum miR-3939 and miR-1910-3p were significantly greater in T2DM-DR patients than in T2DM-noDR patients, but on RT-qPCR validation no statistically significant difference was found for these two miRNAs. The choice of platform for the validation studies is also important. Farr et al. (2015) compared four different high-throughput platforms to validate a circulating miRNA signature for DR. Unprejudiced next generation smallRNA sequencing was carried out to identify a miRNA signature for DR, and the validation of the signature systematically assessed on clinical samples using each of four qPCR platforms which were a standard 96-well platform, a high-content microfluidics platform and two high content platforms. The ViA7 (96-well) platform is the "gold standard" among these qPCR platforms (Hardikar et al., 2014). Five plasma miRNAs (miR-376a-1, miR-132, miR-125b, miR-100, miR-221) were selected based on consistent differences between DR and noDR patients (age and gender matched) and the detection of such a signature was assessed using the four different platforms. The features of miRNA expression were retained across three qPCR platforms - ViA7, TaqMan Low Density Array and Open Array platforms – while the features appeared skewed for the Dynamic Array platform (Farr et al., 2015). Also those microRNAs found to be significantly dysregulated should be tested by ROC analysis and determination of AUC values to establish whether they are good candidates to identify DR and for monitoring disease progression. Further studies are warranted with large cohorts of NPDR and PDR patients and diabetic patients without DR, and of different ethnicities, to be performed in other countries as most of the studies reviewed had been carried out in China.

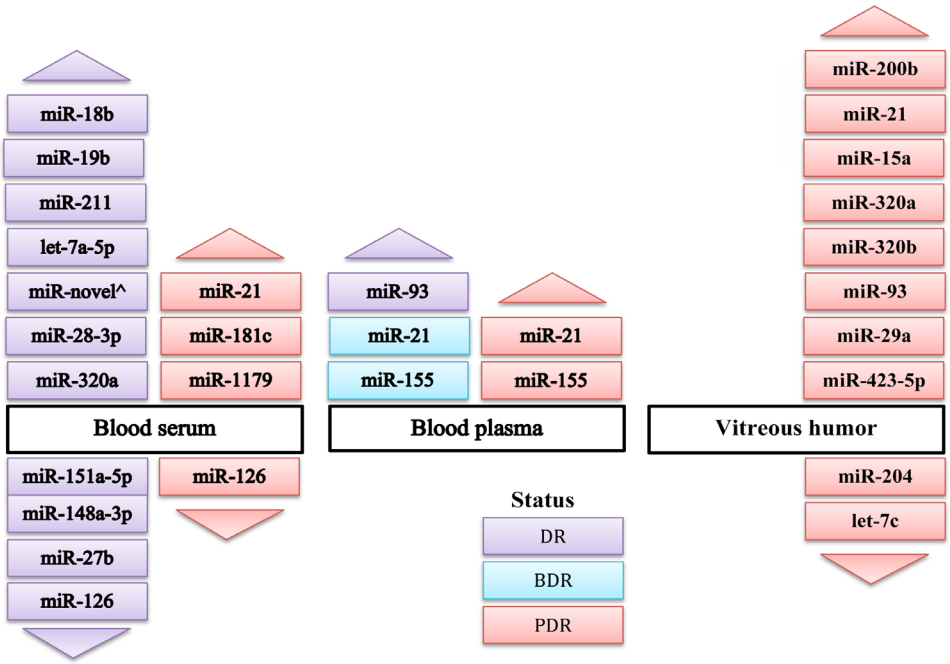

Figure 1 Altered expression of microRNAs in blood serum, blood plasma and vitreous humor of patients with DR.
Blood serum and blood plasma expression levels for DR patients were compared to those of NDR patients, closely matched for age and gender. In the case of PDR, expression levels were compared to those of NDR, NPDR or BDR patients. Vitreous humor expression levels for PDR patients were compared to those of MH patients. Arrows pointing upwards or downwards indicate increased or decreased expression, respectively. miR-novel^ is miR-novel-chr5_15976. DR: Diabetic retinopathy; NDR: no diabetic retinopathy; NPDR: nonproliferative diabetic retinopathy; BDR: background diabetic retinopathy; PDR: proliferative diabetic retinopathy; MH: macular hole.

The biological function of many of the dysregulated miRNAs in DR can be related to effects on retinal cells. The upregulation of miR-211 in DR, and shown in diabetic retinal tissue and hyperglycemic human umbilical vein endothelial cells, could suppress the expression of its downstream target gene SIRT1 *via* specifically binding to the target gene 3′-UTR, leading to retinal vascular disorder and endothelial apoptosis associated with DR (Liu et al., 2018). In addition, the upregulated expression of let-7a-5p in DR may be associated with increased proliferation of retinal microvascular endothelial cells (Liang et al., 2018). The downregulation of miR-126 in DR (Rezk et al., 2016) and PDR (Qin et al., 2017) may be related to endothelial damage as it has been reported that miR-126 provides protection for vascular endothelial cells (van Solingen et al., 2015; Christiakov et al., 2016). MiR-27b and miR-320a, the two miRNAs most consistently associated with the incidence and progression of DR (Zampetaki et al., 2016), have thrombosponin-1 as a target (Stenina-Adognravi, 2013). Thrombosponin-1 inhibits endothelial cell proliferation, migration, and angiogenesis (Tolsma et al., 1997), with its antiangiogenic effect exerted *via* an interaction with VEGF and by inhibiting VEGF receptor-2 signaling (Kaur et al., 2010). The increased expression of miR-21 in DR and PDR (Qing et al., 2014; Jiang et al., 2017) may be associated with it inducing angiogenesis *via* targeting PTEN (phosphatase and tensin homologue), leading to activation of AKT and ERK1/2 signaling pathways, and thereby enhancing HIF-1α and VEGF expression (Liu et al., 2011). Moreover, miR-21 is overexpressed in response to high glucose and protects endothelial cells from apoptosis (Zeng et al., 2013). Also, the increased expression of miR-155 in PDR (Yang et al., 2015) may be related to miR-155 regulating the levels of Treg cells and transforming growth factor-β (Yang et al., 2015). The higher expression of miR-200b in PDR than in MH may be due to miR-200b overexpression downregulating the Oxr1 (oxidation resistance 1) gene, which protects retinal cells against apoptosis and oxidative stress (Murray et al., 2013).

Of interest and clinically important, a very high percentage of patients with eye diseases such as glaucoma, age-related macular degeneration, and DR develop Alzheimer's disease (AD) (Lee et al., 2019). Patients with recent DR (diagnosed within 0–5 years) and established DR (> 5 years) were found to be at a higher risk of AD by 67% and 50% compared to those without DR (Lee et al., 2019). Identifying ophthalmic diseases by measurement of suitable biomarkers would enable better screening and treatment of those individuals at risk of AD (Romano et al., 2017; Lee et al., 2019).

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